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Acute and subchronic benzodiazepine-barbiturate-interactions on behaviour and physiological responses of the mouse

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Abstract. Female NMRI mice were pretreated for 2 weeks with diazepam (D: 20 mg/kg/day), secobarbital (S: 23 mg/kg/day), or combination (D+S: 19 mg/kg/day, each) by means of the drinking fluid. A fourth group remained untreated. One day after this period the mice received an i.p. injection of one out of 16 drug combinations (crossover design: 0, 2, 4, 6 mg/kg D combined with 0, 6, 12, 18 mg/kg S). Open field behaviour, motor performance, and rectal body temperature were measured.

In non-pretreated animals, D and S induced immobility, impairment of coordination and hypothermia in a dose-dependent manner. Excitation appeared after low doses of D (2 mg/kg) and high doses of S (12–18 mg/kg). Acute interactions between D and S were studied by means of isobolographic analysis. Dose-additivity indicating a common mechanism of action was confirmed for immobility, impairment of coordination, and hypothermia whereas excitation revealed a non-additive interaction and was reduced after combined administrations. After chronic pretreatment, the mode of acute drug interaction (dose-additive and non-additive, resp.) remained unchanged. Shifts of the isoboles indicated tolerance, cross-tolerance or sensitization. There was an asymmetry concerning the pretreatment with D and S. Chronic administration of D induced a tolerance to D in regard to all responses and a sensitization to S-effected motor incordination. Chronic administration of S sensitized the sedative and hypothermic responses to acute D. Metabolic tolerance could not account for the subchronic effects since distinct functional responses were concerned in different ways.

The results support the hypothesis that in spite of a partially common mechanism of acute action, chronic adaptation at the $GABA_A$-receptor is differently mediated by barbiturates and benzodiazepines according to regionally and functionally distinct patterns. Excitation contrasts with all the other behavioural of responses. The isoboles indicate separate mechanisms for D and S without any relationship to the sedative effects of the drugs.

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Key words: Barbiturate – Benzodiazepine – Drug interactions – Tolerance – Sensitization – Mouse – Behaviour

Introduction

Analyses of drug interactions may not only provide helpful informations about risk and benefit of therapeutically used drug combinations but can also be used as a tool for basic research on the relationship between drug mechanisms and drug effects. For this purpose several doses of a certain drug are combined with several doses of another one. The dose-response relationships can be described by means of isoboles (lines connecting equally effective dose combinations) (Loewe and Muischnek 1926; Mitchell 1976; Wessinger 1986).

The construction of an isobologram is useful in particular for the discrimination between different kinds of drug interaction. Provided that the drugs share the same mechanism of action, one of them can be substituted by the other one and vice versa. As a consequence, the isoboles are linear; the mode of interaction is called *dose-additive* (Unkelbach and Pöch 1988). In the case that the mechanisms of action of the two drugs are completely different but converge to the same functional response a superposition of effects is expected which can be predicted by the mathematical model of *effect independence* or *effect additivity* (Unkelbach and Pöch 1988; Wessinger 1986).

"Real" drug interactions are neither dose-additive nor effect-independent but non-additive. Potentiation or suppression of one drug's effect by the other one might indicate nested, consecutive, or mutually inhibiting mechanisms. For example, if the two drugs act at different sites within a regulatory loop, a non-additive mode of interaction is probable (Wolffgramm et al. 1988). Consequently, the comparison between observed and expected isoboles provides valuable informations about common, different, or linked mechanisms of drug action.

Both benzodiazepines and barbiturates enhance the chloride flow through the membrane in $GABA_A$-ergic synapses (Harris 1990; Ho and Yu 1991; Morrow et al. 1990; Yu et al. 1988). Hence, they share in part a common mechanism although the molecular sites of action are not identical (Harris 1990; Ho and Yu 1991). This might be the base of a dose-additive interaction. However, some physiological and behavioural responses may be mediated by other mechanisms which do not concern the $GABA_A$ synapse. In this case an independence of effects or a non-additive interaction may be expected. Investigations on physiological and behavioural responses to concomitant administration of a barbiturate and a benzodiazepine may be helpful to close the gap between molecular mechanisms of drug actions and the reactions of the organism.

Most experiments on drug interactions have focussed on acute effects. It has poorly been studied to what extent such interactions are altered by chronic pretreatment. Pharmacodynamic tolerance, cross-tolerance, and sensitization after chronic administration of certain drugs are consequences of physiological adaptation to continuous drug supply. Some of these processes take place on the cellular or molecular level whereas others may be related to drug experience and learning.

An animal model of drug interactions which includes both acute and subchronic drug treatment provides a chance to close the gap between synaptic mechanisms, adaptation processes, and physiological/behavioural responses to the drugs. Based on the hypothesis that a common – or partially common – mechanism of action of the two drugs should not be affected by adaptive regulations taking place during chronic treatment, a dose-additive mode of drug interaction should be maintained. Both tolerance and cross-tolerance toward the other drug are expected to appear (Kalant 1977; Le et al. 1986). An alternative hypothesis predicts non-mutual consequences of subchronic administration of the two drugs assuming that different adaptive processes take place in the synapse after subchronic administration of drugs revealing similar acute actions (Khanna et al. 1988).

The present paper presents data from an experimental series with female mice which received diazepam (D), secobarbital (S) or combinations of both. The mice were pretreated subchronically with one of the two drugs, with the combination, or left drug-naïve. Acute drug administration was performed according to a "rectangular" design of dose combinations. The responses being measured in the experiment concerned body temperature, motor coordination, locomotor depression ("immobility") and "paradoxical" excitation (rapid locomotion).

Methods

Animals and housing. The experiments were performed with female NMRI mice (breeder: Hagemann, Lippische Versuchstierzuchten, Extertal, Germany). Females were chosen because they easily can be kept in groups. Aggressive interactions as seen in groups of male mice have enormous consequences not only for endocrine parameters but also for opioid and GABAergic transmission in the brain. On the other hand, single caging would induce alterations in the GABAergic system, too. The only disadvantage of choosing females is that drug effects may be affected by their oestrus cycle, however this fact only leads to a higher degree of variance but not to systematic deviations. At arrival, the body weights of the mice ranged from 21 to 25 g. After one week, they were randomly divided into four experimental groups according to their drug pretreatment and further on were kept in Makrolon cages ($43 \times 26 \times 15$ cm) with a group size of four individuals per cage. The animals received Altromin R 1320 standard diet ad libitum. Fluid also was provided ad lib., but the content dependent on the experimental condition (water, D solution, S solution, or a combination of S and D; see below). The light/dark cycle was set to 12 h/12 h (light period between 6 a.m. and 6 p.m.). The temperature ranged between 19 °C and 23 °C. Body weight, food consumption, and fluid intake of the mice were registered three times a week. The drinking fluids were completely exchanged twice a week.

Time schedule and drug administration. The period of subchronic drug pretreatment started one week after the arrival of the mice and lasted two weeks. The drugs were diluted in tap water, the resulting solution was offered as the only drinking fluid (see chapter "drugs"). The first experimental group received 100 mg/l D, the second 100 mg/l S, the third one a combination of 100 mg/l D and 100 mg/l S, and the fourth one drug-free water. Since the mice took the drugs by means of self-administration, the daily doses depended on total fluid intake. This mode of subchronic drug administration was used because it guaranteed a rather continuous drug supply during the activity periods of the mice. Furthermore, repeated injections of the drugs would have caused distress reactions and might have induced conditioned responses interfering with the acute drug administrations in the tests.

Behavioural and physiological tests were performed at the end of the pretreatment period after acute administration of S, D, or combinations. Six hours before the tests, the drinking tubes were removed to prevent direct effects of the chronically ingested drug. However, it cannot be excluded that metabolites of diazepam (desmethyldiazepam, oxazepam) are retained in fat tissue even after withdrawal periods of 6–12 h (Van der Kleijn et al. 1971). Any baseline shift after subchronic treatment (response after injection of saline) may possibly be due to such residual benzodiazepines. Since each animal had to pass through three tests (open field, fixed horizontal bar, and body temperature), it was not possible to perform all the tests after one single drug administration. Therefore, acute applications were given twice with two days in between. After one intraperitoneal injection of the drug the open field test was performed, the other administration served for the fixed bar test and the measurement of body temperature. For a given mouse the drug dose was the same on the two days. Half of the animals was first submitted to the open field test and two days later to fixed bar and temperature recording, the others vice versa. By means of statistical comparisons between first-day and second-day measures (Student's t-test) possible influences or repeated drug administration ("acute tolerance") were checked. No significant differences were detected ($P > 0.10$). Therefore, all the measures were combined.

The doses of acute drug administrations were chosen according to a rectangular 4×4 experimental design. 0, 2, 4, and 6 mg/kg D were combined with 0, 6, 12, and 18 mg/kg S. Each of the 16 resulting combinations was administered to 9 mice. The attribution of a dose combination to a certain mouse was randomized. In total, $9 \times 16 \times 4 = 576$ mice were tested. The drugs were diluted in 0.9% saline, the injection volume depended on the body weight (4 µl/g).

Test procedures. The tests were chosen according to the expected actions of benzodiazepines and barbiturates. The open field test was used to study spontaneous locomotion of the mice. The registration took place 1–3 h after the beginning of the dark period. Five min after injection the animal was put into a square arena of 0.5×0.5 m which was subdivided into 16 subsquares. The behaviour of the mouse was recorded on video tape by means of a camera being placed 2 m above the open field. The arena was diffusely illuminated by low intensive white light (0,5 lux). Each registration lasted 10 min.

To assess deficits of motor coordination caused by the drugs, a fixed bar test was performed. The mouse was placed on a horizontal wooden bar (diameter: 8 mm, length: 25 cm, 50 cm above the ground) and left there for 2 min. The task was to stay on the bar for this time interval. One day before drug injection all the mice were trained to fulfill the

task. After the training, untreated mice met the criterion by more than 95%. At test day 5 subsequent measures were taken: the first one directly before injection, the others 10, 20, 30, and 40 min after injection. In each trial, the behaviour of the mouse was scored: *0 points* for dropping off immediately; *1 point* for remaining at the top for less than 1 min; *2 points* for staying there for more than 1 but less than 2 min; *3 points* to meet the criterion completely.

Disturbances of vegetative control were studied by registrating the core temperature of the mice. It was measured by use of a rectal probe connected with a Digimed H11 temperature recorder (range: 24–42 °C). The measures were taken during the inter-test intervals of the fixed bar trials: the first one before drug injection, two others 15 and 35 min after injection.

Data analysis and statistics. The open field recordings were submitted to a time series analysis by means of computerized programs. An observer entered in real time the subsquare crossings of the mouse seen on the video into a computer. The total number of crossings per time is a rather rough measure of sedative or excitatory drug effects because both effects may overlap after administration of a bipolar/biphasic drug. Therefore, we additionally took the distance (in m) travelled by fast square crossings (stays in a subsquare lasting not more than 0.5 s) as a measure for excitation and the total time (in per cent) spent with resting (stays in a subsquare for more than 10 s) as a measure for immobility (sedation).

The fixed-bar test was analysed on the base of the score values. As total measure for the drug effect on motor coordination, the results of the four tests after injection were combined. Apart from this global parameter, the time courses of drug action were assessed by a discriminative analysis of the four subsequent tests. The registration before drug application served as a control. For the recording of body temperature the procedure was similar. The registrations 15 and 35 min after drug injection were compared with the respective pre-injection measure of the same individual. The difference values were used for further analysis.

The first step of statistical analysis was the calculation of mean values and SEM of the above mentioned parameters for every kind of pretreatment and every kind of acute drug administration. The crossover design enabled the calculation of isoboles (lines of identical responses induced by different drug combinations) by means of linear interpolation (Unkelbach and Wolf 1984; Wolffgramm et al. 1988). Separate isobolograms were formed for each kind of subchronic pretreatment.

In order to detect dose-additive interactions the linearity of the isoboles was checked by linear regression analysis and subsequent comparison of the regression line with the 95% confidence limits of the interpolated values. The isoboles expected from effect independence were computed either by an addition of the single drugs' actions (e.g. hypothermic effects) or by a calculation of event probabilities on the base of stochastic independence (Unkelbach and Pöch 1988). In the latter case the probability of incidence of the combination's effect was expected to be the sum of the probabilities for the single drugs minus the product of the two probabilities. For example, if a certain dose of drug A leads to an effect of 60% motor impairment in the fixed bar test and another dose of drug B to 50%, the combination of both should produce $0.6+0.5-0.6\times0.5 = 80\%$. As for dose-additivity, the expected isoboles were compared with the observed ones. Confidence limits of the expected isoboles were estimated according to the rules of error propagation. Significant differences between the effects of (acute or chronic) drug treatment were tested by ANOVA statistics or by nonparametric rank tests. Prerequisites for an ANOVA were normal distributions of the parameters and homogeneity of variances. This criterion was matched by the temperature recordings and the parameters of open field behaviour but not by fixed-bar performance. In the latter case, rank tests were used (Kruskall-Wallis H test and Wilcoxon-Mann-Whitney's U test). In multiple testings the significance level of $P<0.05$ (two-tailed) was adjusted.

Drugs. Secobarbital was taken as pure substance. It was a gift from UCB-Dipha, Brussels, Belgium. For diazepam injections "Diazepam Ratiopharm" ampoules were used. Drinking fluids were prepared with pure diazepam which was a gift from Hoffmann-La Roche, Basel, Switzerland. Since diazepam is not sufficiently soluable in water 2 g ethanol and 0.5 g benzyl alcohol per liter were added. The concentrations of the alcohols were too low to induce any psychotropic effects. Both diazepam and secobarbital are stable in aqueous solutions for several days (Schütz 1986). By means of an enzyme immuno assay technique (modified Emit® assay, Syva Company, USA) no decrease of diazepam concentration was found in drinking solutions kept for 5 days at room temperature.

Results

Housing data

At the beginning of subchronic drug pretreatment body weight, food consumption and fluid intake did not differ among the four experimental groups. During the course of the pretreatment significant differences in body weight and fluid consumption but not in food consumption appeared (Table 1). All mice increased their body weight during the subchronic period, but the increase was higher in mice treated with D or S+D ($P<0.01$, each). The fluid intake of D-treated and, in particular, of S+D-treated mice was lower than that of controls and of S-treated animals. The daily drug doses depended on fluid intake (D: 20.3 ± 1.1 (SD) mg/kg/day; S: 23.3 ± 0.9 mg/kg/day; S+D: 18.5 ± 1.5 mg/kg/day, each). The housing data confirmed that the physical health of the animals was not affected by subchronic drug treatment.

Acute effects of the single drugs

D and S revealed a similar profile of actions in the tests that were performed. The two drugs impaired motor coordination in the fixed bar test, caused hypothermia and – depending on dose and time – induced motor depression and/or excitation in the open field. The effects on fixed-bar performance and body temperature were dose-dependent, i.e. the shapes of the dose-response curves were sigmoid (Fig. 1). Locomotion, however, was affected in a more complex way. With rising doses of S both the

Table 1. Mean values (±SEM) of body weight, food consumption, and fluid intake during drug pretreatment

		Start of experiment	Start of treatment	End of experiment
Body weight (g)	C	24.6±0.15	26.2±0.17	31.1±0.19
	D	24.6±0.16	26.1±0.16	31.8±0.21**
	S	24.5±0.15	26.1±0.17	31.0±0.21
	D+S	24.6±0.14	26.1±0.16	31.9±0.21**
Food consumption (g/day)	C	5.9±0.03	6.1±0.02	6.1±0.02
	D	5.7±0.03*	6.0±0.03	6.2±0.03
	S	5.8±0.03	6.0±0.02	6.2±0.03
	D+S	5.8±0.03	6.1±0.03	6.3±0.03*
Fluid intake (ml/day)	C	6.5±0.04	7.9±0.04	7.2±0.03
	D	6.5±0.03	7.8±0.04	6.4±0.05***
	S	6.4±0.03	7.8±0.03	7.1±0.03
	D+S	6.3±0.04*	7.7±0.04*	5.9±0.7***

C, Controls; D, diazepam-treated; S, secobarbital-treated; D+S, treated with the combination; 144 animals per group, each
Significant differences to controls are marked by asterisks: *$P<0.05$; **$P<0.01$; ***$P<0.001$; ANOVA statistics

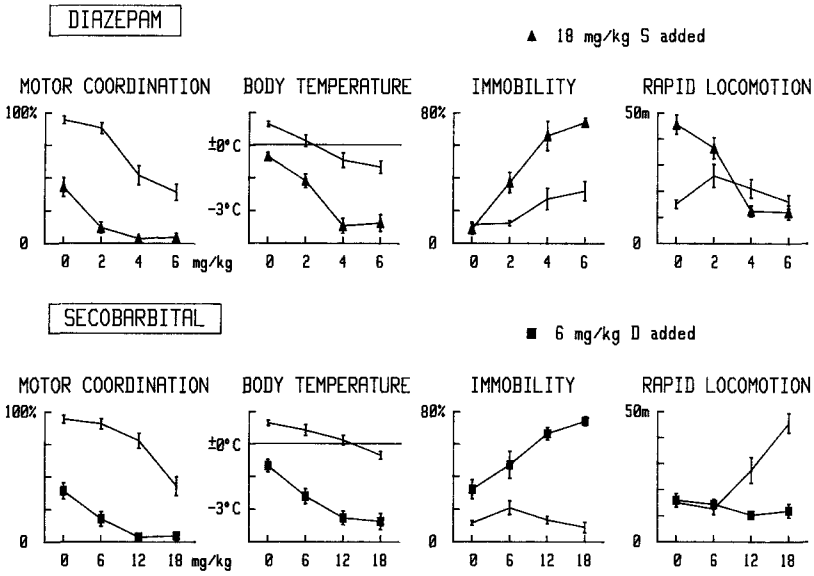

Fig. 1. Dose-response curves of diazepam (*above*) and secobarbital (*below*) according to four responses after acute administration: motor coordination on the horizontal fixed bar (percentage of maximum score, rectal temperature (differences to pre-test values), immobility in the open field (percentage of test duration), and distance travelled in the open field by rapid locomotion (in m). *Missing symbols:* single drugs; *closed symbols:* combined administration of diazepam and 18 mg/kg secobarbital, and of secobarbital and 6 mg/kg diazepam

time spent immobile and the distance travelled with rapid locomotion were increased. Thus, the barbiturate induced sedation as well as excitation by the same does. D also revealed both sedative and excitatory effects, but motor excitation resulted from low doses (2 mg/kg) whereas higher doses caused motor depression (Fig. 1).

The time course of fixed bar performance revealed a maximum effect of both D and S 10–20 min after injection. The performance was completely suppressed by 18 mg/kg S 10 min after administration but restored to 80% after 40 min. The effect of D decreased more slowly. Forty min after administration of the high dose (6 mg/kg) fixed bar performance was still depressed at a level of only 60%.

The hypothermic effect after D or S was in general diminishing from 15 min to 35 min after injection, but with high drug doses the reduction was rather slow (with 6 mg/kg D: −1.2 °C after 15 min, to −1.0 °C after 35 min).

During the open field test of 10 units duration, the animals exhibited a typical time course of motor activity. Locomotion continuously declined with a nearly exponential curve shape. Sedative drug effects did not appear from the beginning but some two minutes after the start of the test. Excitation, measured by an increase of rapid locomotion, was more equally distributed all over the test period.

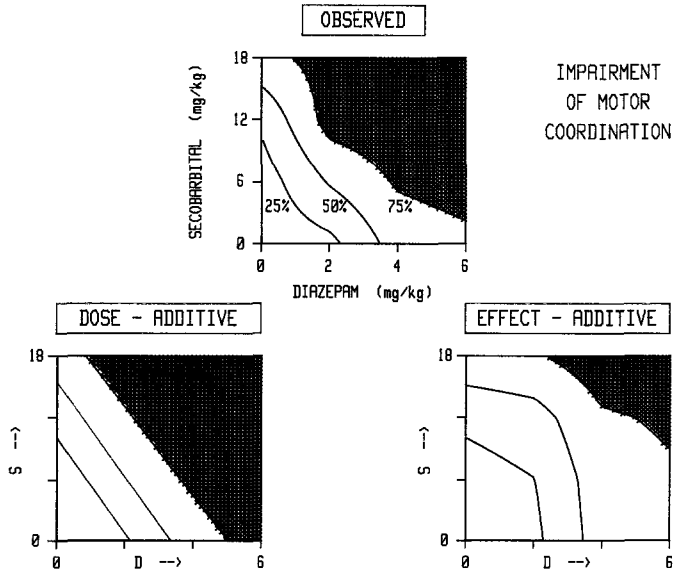

Fig. 2. Impairment of motor performance in the fixed bar test after acute administration of D, S, and combinations. *Above:* observed isobologram including the isoboles of 25%, 50%, and 75% impairment. Dose combinations inducing a response of 75% and more are *hatched*. *Below:* expected isobolograms calculated by means of the mathematical models of dose-additivity (*left*) and effect-additivity (*right*). Observed isoboles differ significantly from effect-additive predictions but not from dose-additivity

Acute interactions between diazepam and secobarbital

The dose-response relationship after concomitant administration of the two drugs can be represented by a battery of dose-response curves (Fig. 1), by a three-dimensional dose-response plot, or by an isobologram (Fig. 2). Motor coordination was synergistically impaired by both drugs. Each drug increased the effect of the other one. The shape of the isoboles was nearly linear (Fig. 2). Deviations from linearity were not significant. This result supported the hypothesis of dose-additivity between S and D. On the other hand the observed isoboles significantly ($P < 0.01$) differed from those predicted by the hypothesis of effect independence (Fig. 2).

A dose-additive synergism of the two drugs represented by linear isoboles was also found in hypothermia and motor sedation (Figs. 6, 7). The respective isoboles significantly differed from those predicted by the hypothesis of effect independence. Excitation, however, revealed a completely different relationship: the isoboles reflected neither dose-additivity nor effect-independence (Fig. 8). Excitation caused by high doses of the barbiturate or by low doses of the benzodiazepine seemed to represent two separate effects which mainly occured after administration

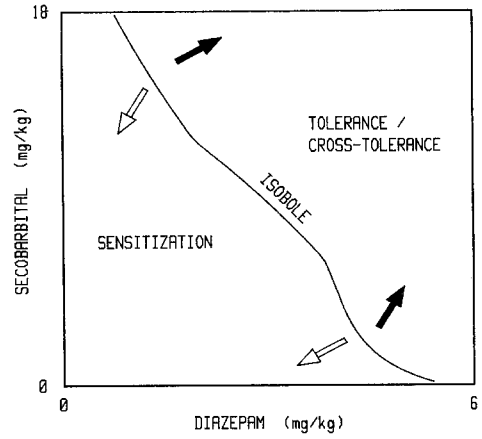

Fig. 3. Displacement of isoboles after chronic drug intake. *Arrows* indicate shifts expected with sensitization (*open arrows*), and tolerance or cross-tolerance (*closed arrows*)

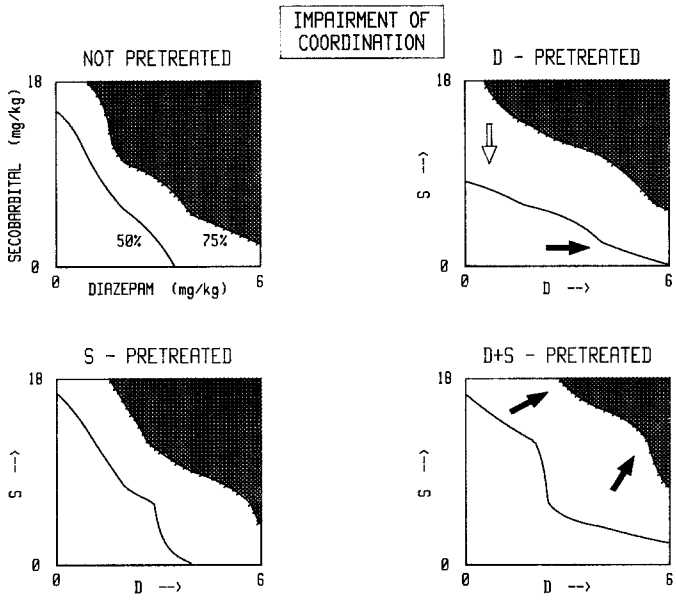

Fig. 4. Impairment of motor performance on the fixed bar. 50% and 75% isoboles are shown for non-pretreated, D-pretreated, and S+D-pretreated mice. Dose combinations inducing a response of 75% and more are *hatched*. *Arrows* indicate significant ($P < 0.05$) displacements of isoboles compared to the non-pretreated group (*open arrow:* sensitization; *closed arrows:* tolerance/cross-tolerance)

of the pure drugs. In combinations excitation was suppressed rather than enhanced. Since the observed isoboles differed significantly from those expected by dose additivity or effect independence the interaction was non-additive.

Effects of subchronic drug pretreatment

Provided that two drugs were acting synergistically in a dose-additive manner after acute administration, similarities in their chronic effects might be expected as well. After a pretreatment period of 2–3 weeks, both tolerance to the administered drug and cross-tolerance to the other drug should appear. This would mean that the isobole lines were shifted towards higher doses, i.e. to the right and to the top (Fig. 3). However, such a symmetry of drug actions did not appear. Instead, pretreatment with D affected fixed-bar performance after acute administrations of S and D in principally different ways. The mice developed a tolerance to D and at the same time a sensitization to S. The isoboles were shifted towards lower doses of S and higher doses of D (Fig. 4). Both tolerance and sensitization were significant ($P < 0.05$, and $P < 0.001$, resp.). The acute interaction between D and S remained dose-additive i.e. the isoboles were still linear. The latter result was true after pretreatment with S as well. In this case a slight, but not significant tolerance to S and cross-tolerance to D was found (Fig. 4). A novel effect appeared after combined drug pretreatment. The mice revealed a high baseline of coordination disturbances which was poorly affected by acute drug administrations. The resulting isobologram revealed an even dose-response area enclosing a wide range between ED_{25} and ED_{75} isoboles (Fig. 5).

The two other drug effects representing dose-additive synergistic interactions between D and S (hypothermia and locomotor depression) also revealed an asymmetrical adaptation to chronic pretreatment. The subchronic effects on hypothermia mainly concerned the acute actions of D. When pretreated with D or D+S the animals revealed tolerance, when pretreated with S they were sensitized to D (Fig. 6). The latter effect was a consequence of a temporal prolongation of D-induced hypothermia. Motor depression was affected in a similar way. After chronic administration of D a tolerance to D and cross-

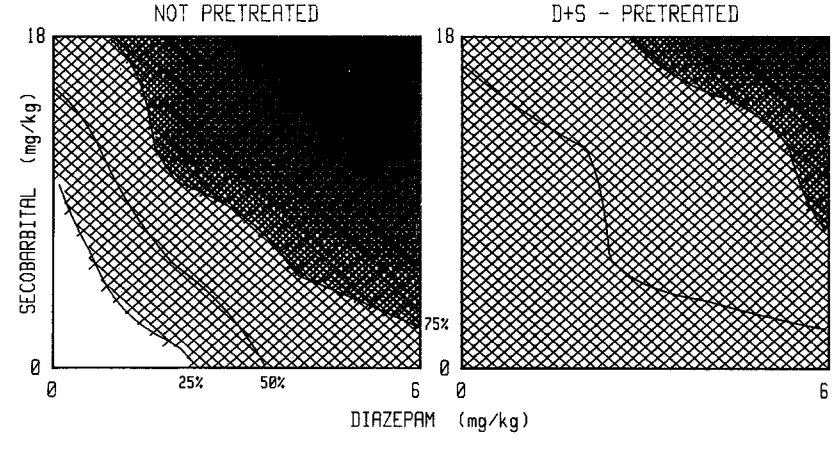

Fig. 5. Impairment of motor coordination on the horizontal fixed bar. 25%, 50%, and 75% isoboles are shown for non-pretreated and D+S-pretreated mice. Responses of 25% and more are hatched, responses of 75% and more are hatched densely. The range between 25% and 75% isoboles is extended after combined treatment

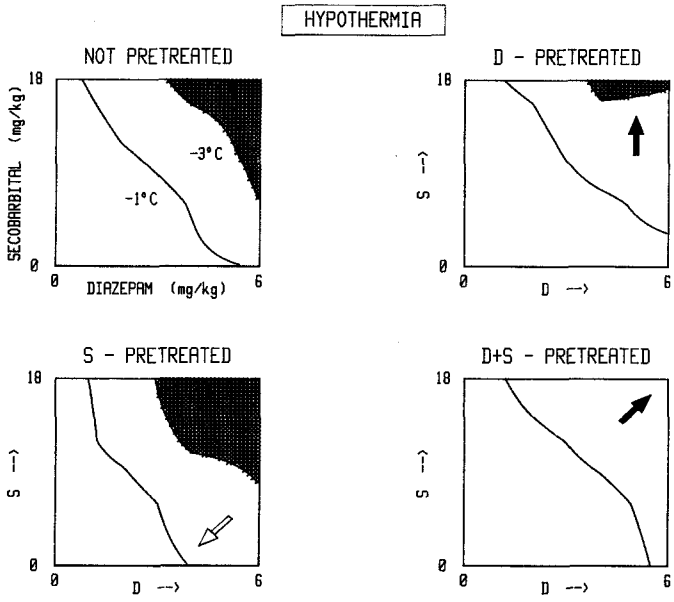

Fig. 6. Isobolograms for hypothermia in non-pretreated and drug-pretreated mice. Isoboles represent a decrease of body temperature of 1 °C and 3 °C, compared to the individual pre-administration values. Hypothermic responses of −3 °C and more are *hatched. Arrows* indicate significant ($P < 0.05$) displacements of isoboles (*open arrow:* sensitization; *closed arrows:* tolerance/cross-tolerance)

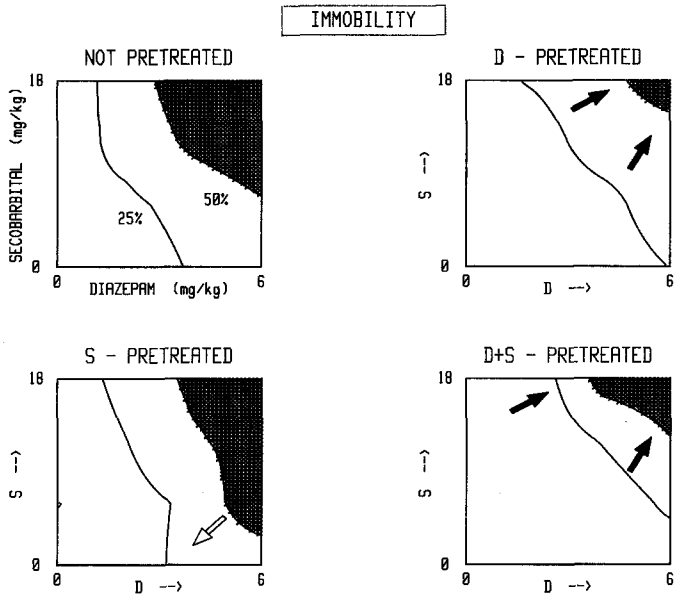

Fig. 7. Isobolograms for immobility in the open field in non-pretreated and drug-pretreated mice. Isoboles represent 25% and 50% of time spent with immobility during the test period. Responses of 50% and more are *hatched.* Arrows indicate significant ($P < 0.05$) displacement of isoboles (*open arrow:* sensitization; *closed arrows:* tolerance/cross-tolerance)

tolerance to S appeared, pretreatment with S led to a sensitization versus D (Fig. 7). In none of these cases the linearity of the isoboles was significantly altered, i.e. the dose-additivity of acute drug interaction remained unchanged.

The non-additive interaction between D and S on motor excitation was poorly affected by drug pretreatment.

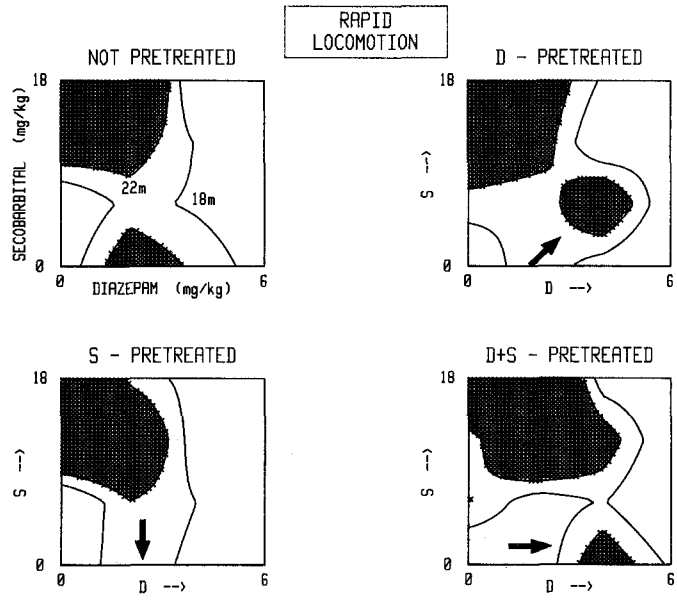

Fig. 8. Isobolograms for rapid locomotion in the open field in non-pretreated mice. Isoboles represent distances of 18 m and 20 m travelled by rapid locomotion. Responses of 22 m and more are hatched. *Closed arrows* indicate significant ($P < 0.05$) displacements of isoboles (tolerance)

The effect of high doses of S was not altered whereas the stimulating actions of low doses of D were attenuated by pretreatment with S and slightly shifted towards higher doses of D by pretreatment with D or D+S ($P < 0.05$, each; Fig. 8). These shifts reflect a tolerance to D. The non-linearity of the isoboles and the non-additivity of interaction were not changed by the subchronic pretreatment.

Discussion

Acute interactions

The study of drug interactions provides a potent means to study common and diverging mechanisms of the actions of two substances in the behaving organism. Linear isoboles indicate common molecular pathways, effect independence suggests separate mechanisms and non-additivity is a hint for functional but not molecular relationships between the drug's actions (Unkelbach and Pöch 1988; Wolffgramm et al. 1988). In the present study, the acute effects of the barbiturate S and the benzodiazepine D on time spent immobile, index of motor coordination, and body temperature but not on excitation were dose-additive irrespective of previous drug pretreatment. This is an indication of a common molecular mechanism being based on an increased chloride influx in $GABA_A$-ergic synapses (Harris 1990; Ho and Yu 1991; Morrow et al. 1990; Yu et al. 1988).

Excitatory actions are typical for both barbiturate and benzodiazepines although they often have been neglected. Their appearance depends on dose, time after administration, and environmental situation (Downes et al. 1970; Gerischer 1990). Some authors considered "para-

doxical" excitation as a secondary consequence of anxiolytic drug properties. They interpreted the increase of locomotion as a reduction of neophobia (Bodnoff et al. 1989). However, there exist marked differences between anxiolytic and excitatory effects of benzodiazepines. The time courses of benzodiazepine-induced anxiolysis and motor stimulation are different. They are differentially affected by benzodiazepine antagonists, excitation is also present in situations without neophobia, and external influences like social deprivation interact in different ways with anxiolytic and excitatory effects (Gerischer 1990; Heyne and Wolffgramm 1990; Phillips and Gallaher 1992). Therefore, excitation seems to be a primary drug action which can clearly be discriminated from anxiolysis. Both benzodiazepines as well as barbiturates are bipolar drugs and motor excitation is an integral part of their spectrum of action.

The results of the present study indicate that the motor stimulations induced by D and by S are two separate phenomena which do not show any dose-additive interactions. It has been known earlier that in both rats and mice excitation is caused by low doses of D and by high doses of S (Gerischer 1990; Wolffgramm et al. 1988). The non-linear shapes of the isoboles indicate different kinds of molecular mechanisms. Therefore, it does not seem likely that GABAergic mechanisms are involved.

It seems likely that regulatory circuits in the brain stem and the limbic system, which control awakeness and vigilance are separately affected by both drugs. Our results are in contradiction to the conclusions of Phillips and Gallaher (1992), who suggested a common biological mechanism mediating the sensitivity to stimulant effects of sedative-hypnotic drugs. However, these authors reported that even stimulation by two benzodiazepine-compounds was differentially blocked by the benzodiazepine antagonist flumazenil. Therefore a common mechanism seems unlikely. The result that excitations elicited by certain bipolar drugs do not superimpose but rather attenuate each other is not unique. It might reflect a general principle that combinations of bipolar drugs induce less excitation than the single substances do (Hu et al. 1986; Wolffgramm et al. 1988). As a consequence, the balance between excitation and sedation is shifted towards of sedation.

Subchronic interactions

Chronic drug treatment induces regulatory adaptations which can be described by negative feed-back loops. In many cases synaptic adaptations are well correlated to functional ones (Miller et al. 1989; Rommelspacher et al. 1989; Saunders et al. 1990; Trullas et al. 1987; Wolffgramm et al. 1990). Although some authors failed to detect parallels between synaptic and physiological responses to chronic benzodiazepine treatment (Heninger and Gallager 1988), there have been several studies reporting that pharmacodynamic tolerance and decrease of benzodiazepine receptor binding develop simultaneously (Miller et al. 1988). As a consequence, the facilitation of GABA-induced chloride influx by benzodiazepine is reduced after chronic treatment. Such desensitization seems to be restricted to the benzodiazepine binding site. Effects of GABA are not affected (Yu et al. 1988).

Supposed that two drugs reveal dose-additive interactions and are therefore likely to share at least in part a common mechanism, it should be expected that their chronic effects do as well. Such a symmetry resulting in mutual tolerance and cross-tolerance was not found. A similar kind of asymmetry concerning cross-tolerance between pentobarbital and ethanol has been reported by Khanna et al. (1988). On the one hand, the lack of symmetry concerned the effect of a certain pretreatment on the acute actions of S and D. Impairment of coordination caused by D, S, or combinations was still dose-additive after pretreatment with D, but the acute effect of D was attenuated whereas that of S was even sensitized. On the other hand, the chronic effects of D and S were asymmetrical, too. Administration of S for two weeks did not cause any sensitization at all. Instead, both slight tolerance and cross-tolerance towards both drugs appeared. Therefore, the sites of chronic adaptations are not likely to be the same for barbiturates and benzodiazepines. There are two possible explanations. Either adaptive regulation takes place in the $GABA_A$-ergic synapse itself or other transmission systems are concerned. The existence of acute in-vivo interactions between barbiturates and benzodiazepine receptor binding might provide a basis for long-term regulation processes (Saunders et al. 1990). Recent research on adaptive regulation in $GABA_A$-ergic synapses indicates that such chronic processes do not always concern the whole $GABA_A$ receptor complex but may affect distinct subunits and binding sites in different ways (Ngur et al. 1990; Ramsey et al. 1991; Yu et al. 1988).

In this study, the consequences of subchronic pretreatment for sedative, hypothermic, and coordination-impairing effects were not qualitatively and quantitatively identical. There were good similarities concerning the results on immobility and hypothermia but not on motor coordination. This discrepancy indicates that tolerance cannot be due to metabolic adaptations but must be a consequence of functional tolerance probably taking place at the pharmacodynamic level. Conditioned tolerance to the drug can be excluded, since the routes of acute and chronic drug administration were different. It is known that $GABA_A$ receptors in different areas of the brain consist of different protein subunits (Massotti et al. 1991; Sigel et al. 1990). Chronic pretreatment may affect such receptors in different ways (Ho and Yu 1991; Morrow et al. 1990). For instance, enhancement of GABA-stimulated chloride influx is reduced in cortical neurons of rats after chronic pretreatment with diazepam whereas no comparable effects can be observed in the cerebellum (Marley and Gallager 1989).

An interesting phenomenon being potentially important for long-term intake of drug combinations is the interaction of chronic intake of D+S on coordination impairment. A marked baseline shift coincided with a tolerance to both D and S. As a consequence, the increased level of coordination impairment remained nearly unaffected by acute administrations of the two drugs or of combinations. A probable explanation is an increased

susceptibility to stressors. Just before the injection of saline, D+S pretreated mice revealed a normal (undisturbed) motor performance, but after injection their coordination was impaired although they did not have received any drug. Non-pretreated mice were not affected by saline injection. Therefore, pretreatment with psychotropic drugs – in particular with the drug combinations used – may interfere with the individual's capability to cope with stress deriving from environmental stimuli.

Chronic drug intake is altering the responses to excitatory and sedative actions of psychotropic drugs. The balance between excitation and sedation has been suggested to be responsable for the reward following the intake of a psychotropic drug (Coper et al. 1990; Wolffgramm 1991). Therefore, drug combinations may bear additional risks of abuse and dependence compared with their single components.

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References

Bodnoff SR, Suranyi-Cadotte BE, Quirion R, Meaney MJ (1989) Role of the central benzodiazepine receptor system in behavioral habituation to novelty. Behav Neuroscience 103:209–212

Coper H, Rommelspacher H, Wolffgramm J (1990) The 'point of no return' as a target of experimental research on drug dependence. Drug Alcohol Depend 25:129–134

Downes H, Perry RS, Ostlund RE, Karler R (1970) A study of the excitatory effects of barbiturates. J Pharmacol Exp Ther 175:692–699

Gerischer C (1990) Environmental interactions to excitation and sedation in mice after application of diazepam, caffeine and combinations. Naunyn Schmiedebergs Arch Pharmacol (Suppl) 341:R 101

Harris RA (1990) Distinct actions of alcohol, barbiturates and benzodiazepines on GABA-activated chloride channels. Alcohol 7: 273–275

Heninger C, Gallager DW (1988) Altered gamma-aminobutyric acid/benzodiazepine interaction after chronic diazepam exposure. Neuropharmacology 27:1073–1076

Heyne A, Wolffgramm W (1990) Avoidance behavior against an anxiogenic stimulus: interaction between social deprivation and anxiolysis. In: Elsner N, Roth G (eds) Brain – perception – cognition. Thieme, Stuttgart, p 442

Ho IK, Yu S (1991) Effects of barbiturates on GABA system: comparison to alcohol and benzodiazepines. Keio J Med 40:183–186

Hu WY, Reiffenstein RJ, Wong L (1986) Interaction between flurazepam and ethanol. Alcohol Drug Res 7:107–117

Kalant H (1977) Comparative aspects of tolerance to, and dependence on, alcohol, barbiturates and opiates. In: Gross MM (ed) Alcohol intoxication and withdrawal III. Plenum Press, New York, pp 169–186

Khanna JM, Le AD, Gougos A, Kalant H (1988) Effects of chronic pentobarbital treatment on the development of cross-tolerance to ethanol and barbital. Pharmacol Biochem Behav 31:179–186

van der Kleijn E, van Rossum JM, Muskens ETJM, Rijntjes NVM (1971) Pharmacokinetics of diazepam in dogs, mice and humans. Acta Pharmacol Toxicol 29, Suppl 3:109–127

Le AD, Khanna JM, Kalant H, Grossi F (1986) Tolerance to and cross-tolerance among ethanol, pentobarbital and chlordiazepoxide. Pharmacol Biochem Behav 24:88–98

Loewe S, Muischnek H (1926) Über Kombinationswirkungen. 1. Mitteilung: Hilfsmittel der Fragestellung. Naunyn Schmiedebergs Arch Exp Path Pharmakol 114:313–326

Marley RJ, Gallager DW (1989) Chronic diazepam treatment produces regionally specific changes in GABA-stimulated chloride influx. Eur J Pharmacol 159:217–223

Massotti M, Schlichting JL, Antonacci MD, Giusti P, Memo M, Costa E, Guidotti A (1991) Gamma-aminobutyric $acid_A$ receptor heterogeneity in rat central nervous system: studies with clonazepam and other benzodiazepine ligands. J Pharmacol Exp Ther 256: 1154–1160

Miller LG, Greenblatt DJ, Barnhill JG, Shader RT (1988) Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased gamma-aminobutyric $acid_A$ receptor function. J Pharmacol Exp Ther 246:170–176

Miller LG, Woolverton S, Greenblatt DJ, Lopez F, Roy RB, Shader RI (1989) Chronic benzodiazepine administration. IV. Rapid development of tolerance and receptor downregulation associated with alprazolam administration. Biochem Pharmacol 38:3773–3777

Mitchell CL (1976) The design and analysis of experiments for the assessment of drug interactions. Ann NY Acad Sci 281:118–135

Morrow AL, Montpied P, Lingford-Hughes A, Paul SM (1990) Chronic ethanol and pentobarbital administration in the rat: effects on $GABA_A$ receptor function and expression in the brain. Alcohol 7:237–244

Ngur DO, Rosenberg HC, Chiu TH (1990) Modulation of GABA-stimulated Cl^- flux by a benzodiazepin agonist and an 'inverse agonist' after chronic flurazepam treatment. Eur J Pharmacol 176:351–356

Phillips TJ, Gallaher EJ (1992) Locomotor responses to benzodiazepines, barbiturates and ethanol in diazepam-sensitive (DS) and -resistant (DR) mice. Psychopharmacology (Berlin) 107:125–131

Ramsey VA, Tietz EI, Rosenberg HC (1991) Chronic flurazepam differentially regulates a behavioral effect of GABA agonists. Pharmacol Biochem Behav 38:659–663

Rommelspacher H, Wolffgramm J, Widjaja S (1989) Effects of desipramine on rat behavior are prevented by concomitant treatment with ethanol. Pharmacol Biochem Behav 32:533–542

Saunders PA, Ho Y, Baker ML, Hume AS, Ho IK (1990) Pentobarbital tolerance and withdrawal: correlation with effects on the $GABA_A$ receptor. Pharmacol Biochem Behav 37:343–348

Schütz H (1986) Dünnschichtchromatographische Suchanalyse für 1,4 Benzodiazepine in Harn, Blut und Mageninhalt. VCH Verlagsgesellschaft, Weinheim, Germany

Sigel E, Baur R, Trube G, Mohler H, Malherbe P (1990) The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. Neuron 5:703–711

Trullas R, Havoundjian H, Zamir N, Paul S, Skolnick P (1987) Environmentally-induced modification of the benzodiazepine/GABA receptor coupled chloride ionophore. Psychopharmacology (Berlin) 91:348–390

Unkelbach HD, Pöch G (1988) Comparison of independence and additivity in drug combinations. Arzneim Forschg/Drug Res 38:1–6

Unkelbach HD, Wolf T (1984) Drug combinations-concepts and terminology. Arzneim Forschg/Drug Res 34:935–936

Wessinger WD (1986) Approaches to the study of drug interactions in behavioral pharmacology. Neurosci Biobehav Rev 10:103–113

Wolffgramm J (1991) An ethopharmacological approach to the development of drug addiction. Neurosci Biobehav Rev 15:515–519

Wolffgramm J, Lechner J, Coper H (1988) Interaction of two barbiturates and an antihistamine on body temperature and motor performance of mice. Arzneim Forschg/Drug Res 38:885–891

Wolffgramm J, Rommelspacher H, Buck E (1990) Ethanol reduces tolerance, sensitization and up-regulation of D2-receptor after subchronic haloperidol. Pharmacol Biochem Behav 36:907–914

Yu O, Chiu TH, Rosenberg HC (1988) Modulation of GABA-gated chloride ion flux in rat brain by acute and chronic benzodiazepine administration. J Pharmacol Exp Ther 246:107–113